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(54) Title: MANNOSYLATED PEPTIDES

(57) Abstract

T cells mediate specific immune reactions. They recognize (foreign) protein fragments – peptides – that are bound in the peptide binding groove of MHC molecules. These peptide MHC complexes are generated intracellularly and subsquently transported to the cell surface for recognition by T cells. A rate limiting step in the MHC restricted presentation of (foreign) peptides derived from extracellular sources is therefore the uptake of such antigens by antigen presenting cells and the delivery to the intracellular compartment(s) where the MHC-peptide complexes are formed. Professional antigen presenting cells like dendritic cells and macrophages take up antigens by macropinocytosis and mannose receptor mediated endocytosis. We have explored the possibility that the mannosylation of otherwise non-mannosylated antigens would lead to enhanced uptake and MHC restricted presentation of (fragments of) such antigens by mannose receptor positive cells. Using mannose receptor positive cultured human dendritic cells as antigen presenting cells we found that the mannosylation of antigenic peptides resulted in a 300 – 10.000 fold enhanced potency to stimulate MHC class II restricted peptide-specific T cell clones compared to non-mannosylated peptides. These results indicate that mannosylation of antigens leads to selective targeting and subsequent superior presentation by dendritic cells, a result which may be applicable in for example vaccine design.

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Title: Mannosylated Peptides

The present invention relates to the field of immunology, in particular cellular immunology. More specifically it relates to the presentation of antigen-derived peptides on the surface of antigen presenting cells (APC's) in the context of an MHC-molecule.

T cells play a central role in the immune system. They mediate specific immune reactions against pathogens and tumors but also cause autoimmune diseases and are responsible for the initiation of transplant rejection. Research in the last ten years has revealed that T cells recognize protein fragments - peptides - that are bound in the peptide binding groove of MHC-molecules. These peptide MHC-complexes are generated intracellularly and subsequently transported to the cell surface in order to allow detection by T cells. Peptides derived from intracellularly synthetized proteins most commonly are found in association with MHC-class I molecules. Peptides derived from endocytosed proteins synthetized outside the cells are most commonly found in association with MHC-class II molecules. It is well established that primary T cell responses are initiated most efficiently if peptides are presented by MHC-molecules on a specialized class of antigen presenting cells: cells of the dendritic lineage. Dendritic cells are present in low numbers in peripheral blood, in lymphoid tissues and epithelia. Their extraordinary capacity to induce primary immune responses is attributed to the high cell surface expression of MHC-class I and class II molecules, adhesion molecules and high co-stimulatory activity. Other, currently unknown proteins may also contribute to this process.

One of the problems encountered in trying to induce T cell responses to proteinaceous antigens is that peptides are not efficiently presented by APC's. This problem may lie in the uptake of the proteinaceous antigen or a peptide

derived therefrom by the APC, in the processing of the antigen (cleavage sites and/or transport signals for transport to the right compartment and eventually the surface of the APC, in the complexing of the peptide with the MHC-molecule, etc). Any of these problems may be the reason why peptide or protein based vaccines for many specific pathogens have met with limited success. The present invention indentifies an important limiting step in antigen processing. Said limiting step is the uptake of proteinaceous antigens or peptides derived therefrom.

The present invention improves the uptake of said proteinaceous antigens or peptides by APC's by providing said antigen or peptide with one or more carbohydrate moieties which has an affinity for a mannose receptor on the surface of an APC.

Recent reports have revealed that both cultured monocyte-derived human dendritic cells and murine dendritic cells in situ express mannose receptors and that this mannose receptor is used for the efficient endocytosis of mannosylated proteins.

It has been shown that DCs play a critical role in antigen presentation in vivo and that they can prime virgin T cells (R.M. Steinman, Annu. Rev. Immunol. 9, 271-296 (1991). It has also been shown that receptor-mediated uptake of antigens can effect antigen presentation via MHC class II (A. Lanzavecchia, Annu. Rev. Immunol. 8, 773-793 (1990)). Recently, F. Sallusto et al. (J. Ex. Med. 182, 389-400 (1995)) have shown that endocytosis of antigens by DCs occurs in various ways including fluid phase uptake, macropinocytosis and MR-mediated uptake, and that macromolecules that are endocytosed by one of the latter two ways of receptor-mediated endocytosis are concentrated in the MHC class II compartment. R.M. Steinman and J. Swanson (J. Exp. Med. 182, 283-288 (1995)) raised the question whether DCs might have receptors that mediate absorptive uptake and deliver these as peptides to MHC products. However, no

experimental data have been presented in this publication to verify the hypothesis. A. Lanzavecchia (Curr. Opion Immunol. 8, 348-354 (1996)) has reported that DCs can present low concentrations of soluble antigen by endocytosis via macropinocytosis and that the complexing of antigens with antibodies and the mannosylation of antigens can be employed to target antigens or toxins to dendritic cells. No mention is made of the possibility of enhancing antigen presentation on dendritic cells by targeting the mannose receptor. The statements are based on personal communications and no experimental data, like the nature of the antigens and the way of mannosylation are presented. European Patent Application 0 659 768 A2 (publication date 28.06.1995) describes various *in vivo* experiments with conjugates of peptides/proteins with a carbohydrate polymer (especially mannan). Immune responses could be measured following treatment with a conjugate of a fusion protein with a carbohydrate polymer in mice. No data were presented on the effectiveness of protein-carbohydrate monomer conjugates nor on that of conjugates of peptides with carbohydrate monomers or polymers. There is no disclosure on the mannose receptor or its use for enhancing antigen presentation.

Thus the invention provides a method for enhancing uptake of a proteinaceous antigen or a peptide derived therefrom by antigen presenting cells having a mannose or similar receptor, whereby the antigen is provided with at least one and preferably at least two mannose groups or a functional equivalent thereof. In the context of this application it is clear that (partial) agonistic peptides and antagonistic peptides are useful (as also explained in the experimental part) to be presented to T cells. In the one case the response can be enhanced, in the other case blocked. Both kinds of peptides may be mannosylated themselves or may be part of larger mannosylated structures, such as proteinaceous "antigens".

An important aspect of the present invention is that the antigen or peptide is targeted to a mannose receptor present on APC's such as the mannose receptor present on dendritic cells. For this purpose a mannose group (or more than one) attached to the antigen or the peptide is of course very suitable, but other glycosyl groups may also be used as long as they have a significant binding affinity for a mannose receptor. The structure of the total glycosyl group is not overall important, as long as at least one carbohydrate moiety is recognized by the mannose receptor. The number of glycosylic residues may thus also vary. Our experiments show that the number of carbohydrates attached to the peptide (n=1-6 or preferably 2-6) only marginally influences the presentation of the peptide on the cell surface. It is clear that the use of a different number of carbohydrate moieties on the peptide (n>6) than the number we used will show a comparable effect. Other moieties that have binding affinity for mannose receptors on APC's include, but are not limited to b-L-Fucose, b-D-Glucopyranose, b-D-N-Acetylgalactosamine, etc. This holds true also for modified carbohydrates which have affinity for the mannose receptor like carbohydrate analogs possessing instead of an oxygen atom, an anomeric carbon or an anomeric sulfur atom. It could be a derivative containing one or more halogen atoms replacing one or more hydroxyl(s). It may be modified carbohydrate derivatives with seven, eighth or more atom rings.

The improvement in the uptake by targeting a mannose receptor and the transport by said mannose receptor to the right compartment in the cell for processing, and/or complexing to an MHC-I or MHC-II molecule results in a very efficient presentation of the mannosylated peptide on the surface of the APC's, in particular those of dendritic origin.

Our results demonstrate that mannosylation of peptides leads to very efficient uptake and presentation of these peptides by dendritic cells at low concentrations of peptide. Depending on the T cell clone used, 300 - 10,000 fold less

mannosylated peptide is required for the induction of T cell proliferation when compared with non-mannosylated peptides. Furthermore, only a short exposure to mannosylated peptide is required for the generation of sufficient MHC-peptide complexes to allow the induction of T cell proliferation. Similarly we demonstrate that 1,000 fold less of a mannosylated peptide with antagonistic properties is required for the induction of T cell non-responsiveness when compared to non-mannosylated peptide. This indicates that the mannose receptor expressed on the cell surface of cultured dendritic cells mediates very efficient transport of mannosylated peptides to the intracellular compartment were (newly synthetized) MHC-class II molecules bind peptides. We also observed that mannosylated peptides are presented more efficiently than non-mannosylated peptides when human peripheral blood mononuclear cells are used as antigen presenting cells. This shows that enough mannose receptor positive antigen presenting cells are present in these peripheral blood preparations to allow enhanced presentation. It is known that in peripheral blood approximately 1% of the cells are immature dendritic cells that could mediate this effect.

In the peptides we used, the carbohydrate moieties were attached to the N-terminus of the peptides. It is clear that the same biological effect would be obtained with peptides in which the carbohydrate moieties are attached to the C-terminus, or to parts of the peptides between the N-terminus and the C-terminus e.g. to side chains. Also peptides having carbohydrate moieties attached to combinations of the possibilities mentioned above are expected to have the same biological effect.

It is clear that peptide-like compounds (peptoids and/or peptidomimetics) to which carbohydrates are attached can have the same effect. Peptoids could be peptide-like compounds having a modified backbone structure because they contain unnatural amino acids, D-amino acids, amino acid like building blocks such as ß....½ -amino acids, amide bond

mimics like reduced peptide bonds, sulfonamide bonds, sulfinamide bonds, ester bonds, ether bonds, disulfide bonds, thioether bonds or saturated an unsaturated carbon-carbon bonds. Peptoids could be PNA also.

It is unlikely that the way the glycosylic groups are attached to the peptides is of crucial importance for the biological effect. Other ways of attaching the glycosylic group(s) could be the reaction of other amine reactive groups in the glycosylic moiety like active esters, acid chlorides, acid bromides, acid iodides, symmetric anhydrides, unsymmetric anhydrides, in situ preactivated carboxylic acids to amines in the peptides. It could be the reaction of activated carboxylic groups in the peptide to an amine in the glycosylic moiety. It could be the reaction of a thiogroup in the peptide to a thioreactive group in the carbohydrate moiety like an S-Npys, an S-Nps, a thiol, an acetylbromide, an acetyliodide. It could be the reaction between a thiogroup in the carbohydrate to a thiolreactive group in the peptide, like an acetylbromide, an acetyliodide. It could be an ester forming reaction between a activated carboxylic acid, an acid chloride, an acid bromide, an acid iodide, an in situ activated carboxylic acid in the carbohydrate moiety and a hydroxy group in the peptide. It could be any nucleophilic substitution reaction between a modified carbohydrate and a suitable reactive center in the peptide. It could be any addition reaction of a modified carbohydrate and a suitable reactive center in the peptide.

It is evident that this type of compound could be synthesized completely by solid phase strategies. Such compounds could be synthesized by performing peptide synthesis using amino acids bearing carbohydrate moieties in the side chain. These glycopeptides could be made by using amino acids functionalyzed on the a-amino group with carbohydrate moieties that are introduced at the N-terminus of the peptide to a branched cluster of amino acids possessing various amino functionalities.

Multiple glycosylic moieties in the peptide could also be attached to the peptide by a dendrimer type of structure. These compounds can be synthesized by using bis-amino acids (like lysine, ornithine and diaminopropionic acid). During synthesis the protecting groups are removed from both amino groups before coupling of the next amino acid. This procedure yields constructs with a branched structure, with the possibility to couple glycosylic moieties to the branches.

The best results will of course be obtained if the peptides which are provided to the mannose receptor or which are the result of processing of the proteinaceous antigen delivered to the mannose receptor also have the other characteristics favorable to APC presentation in the context of MHC-class I or MHC-class II molecules. These include, the right size to fit on the MHC-molecule, the right anchor residues to fit in the respective MHC-molecules, transport signals for arriving at the MHC-molecule and for the proteinaceous antigen the proper cleavage sites for the processing enzymes of the APC's.

The mannosylated proteins and/or peptides, possibly in combination with mannose receptor expressing dendritic cells or dendritic-like cells, can be used for a number of purposes. These include, but are not limited to:

- The use of mannosylated proteins and/or peptides for vaccination purposes. Amongst others this includes vaccination protocols for the induction of immunity against pathogens like (myco)bacteria, virusses, yeasts, fungi, helminths and parasites but also vaccination protocols designed to enhance or elicit tumor specific responses.

  For instance desired mannosylated (peptide) antigens may comprise (mixtures or combinations of) known antigenic proteins or peptides, or one or more covalently linked known T helper and cytotoxic T cell epitopes. For example, a known T helper cell epitope that evokes a memory response and thus may aid in eliciting a CD8 response to the

cytotoxic T cell epitope, for example a tumor specific peptide.

Another delivery system may utilize mannosylated liposomes or vesicles into which the desired antigenic proteins or peptides have been incorporated.

- T cells reactive with autoantigens have been described and are thought to play a role in the development of autoimmune diseases like type I diabetes and reumathoid arthritis. Similarly, alloreactive T cells, responsible for graft rejection after organ transplantation and the development of graft versus host disease after bone marrow transplantation, have been shown to be often specific for or dependent on the presence of particular MHC bound peptides. Immune intervention can be envisioned at the level of competition for binding to MHC molecules. For this purpose synthetic compounds with high affinity for the peptide binding groove must be designed that are able to compete for binding to MHC molecules and thus block the binding of immunogenic autoantigen derived peptides to these MHC molecules. This approach is hampered by the relative inefficient uptake of peptides by antigen presenting cells and can be facilitated by the use of mannosylated peptides.

- The use of mannosylated peptides with antagonistic properties for the induction of T cell non-responsiveness. Peptides with antagonistic properties have been described. These peptides differ from the agonist in the nature of the amino acid(s) at T cell receptor contact points and can (partially) block the response to the agonist. The in vivo application of peptides with antagonistic properties may be hampered by the excess of antagonist peptide that is usually required to achieve efficient blocking of the T cell response and the short half-life of peptides in vivo. The use of mannosylated peptides with antagonist properties may be applicable for the efficient targetting of the peptides to

dendritic cells and other mannose receptor positive cells and thus escape from degradation and more efficient loading on MHC molecules, leading to the induction of T cell non-responsiveness at much lower peptide concentrations.

Vaccines may of course contain usual additives and/or adjuvantia and/or carriers as may other pharmaceutical preparations.

The invention will be explained in more detail in the following experimental part.

EXPERIMENTAL PART

Introduction

In our experiments we used various mono- and multiple-mannosylated peptides in which the mannose groups had been attached to the N-terminus of the peptides. For this we synthesized peptides containing in addition an N-terminal lysine and peptides containing in addition various N-terminal lysines (n=1-5).

Mannosylation was performed by reaction of the N-terminal amine group and the amine group(s) in the side chains of the lysine(s) with an isothiocyanate derivative of mannose, yielding an thioureum linkage between the peptide and the mannose moieties.

Herein we use the word mannose frequently. In the experiments the word mannose should be read as mannose, everywhere else the word mannose should be read as "every carbohydrate moiety that shows affinity for the mannose receptor".

The formation and expression of antigenic MHC-peptide complexes was measured using pro

liferative peptide specific T cell clones. The clones were cultured together with HLA-DR matched cultured dendritic cells in the presence or absence of a concentration range of the appropiate peptide. The peptides were either non-mannosylated or mannosylated. Activation of the T cell clones

was measured by the incorporation of [^{3}H]-thymidine after 48 hours of culture. In these experiments we observed that 300- to 10.000 fold less of the mannosylated peptide was required for the induction of half-maximal proliferation compared to the non-mannosylated peptide.

MATERIALS AND METHODS

Synthetic peptides

Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) (Gausepohl et al., 1990[a]). TentagelS AC (Rapp, Tübingen, Germany), (Sheppard et al., 1982; Rapp et al., 1990), a graft polymer of polyethyleneglycol and polystyrene to which the C-terminal amino acid of the peptide had been attached by the manufacturer, was used as a resin (loading 0.2 μeq, particle size 90μm). Repetitive couplings were performed by addition of a mixture of 90 μl 0.67 M PyBOP (Gausepohl et al., 1990[b]; Coste et al., 1990) in NMP, 20 μl NMM in NMP 2/1 (v/v) and 100 μl of a 0.60 M solution of the appropriate Fmoc amino acid (Fields et al., 1990) in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 μl dichloromethane were added to each reaction vessel. Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA 1/4 (v/v) to each reaction vessel. Coupling- and deprotection times were increased as the synthesis proceeded, starting with 30 min and 3 times 3 min, respectively. Washings after coupling and Fmoc-deprotection steps were done with 6 times 1.2 ml DMA. After synthesis the peptidylresins were washed extensively with DMA, dichloromethane, dichloromethane/ether 1/1 (v/v) and ether respectively, and dried in the air (at least 2 h). Cleavage of the peptide and removal of the side chain protecting groups was performed by adding 6 times 200 μl TFA/water 19/1 (v/v) at 5 min intervals to each reaction vessel. 2.5 h after the first TFA addition the peptides were

precipitated from the combined filtrates by the addition of 10 ml ether/pentane 1/1 (v/v) and cooling to -20_C. The peptides were isolated by centrifugation (-20_C, 2,500g, 10 min). After titration of the pellet with 10 ml ether/pentane 1/1 (v/v) and isolation by the same procedure, the peptides were dried at 40_C for 15 min. Each of the peptides was dissolved in 2 ml water (or 2 ml 10 vol% acetic acid), the solution frozen in liquid nitrogen for 3 min, and lyophilized while being centrifuged (1,300 rpm, 8-16 h). The peptides were stored at -20_C until use. The purity of the peptides was determined by analytical reversed phase HPLC using a water-acetonitrile gradient containing 0.1 % TFA, and proved to be at least 70% pure (UV 214 nm). The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) on a Lasermat mass spectrometer (Finnigan MAT, UK). About 5 pmol of the peptide in 0.5 µl water/acetonitrile containing 0.1% TFA was mixed with 0.5 µl of matrix solution (ACH, 10 mg/ml in acetonitrile/water 60/40 (v/v) containing 0.1% TFA) and applied to the instrument. Calibration was performed with peptides of known molecular mass (1422.7 or 1896.1), either as external or as internal references.

Attachment of the carbohydrate moieties to the peptides

In the procedure described below a-D-mannopyranosyl-phenylisothiocyanate was used for the introduction of the mannose moieties in the peptides. The same procedure was followed for the synthesis of the peptides containing other glyco moieties.

To a solution of 2 mg of a synthetic peptide in 200 µl DMSO was added 2 mg of a-D-mannopyranosylphenylisothiocyanate (Cas 96345-79-8) per amino group in the peptide and 1 µl of NMM per amino group in the peptide, respectively.

The reactionmixture was stirred overnight, afterwhich the excess a-D-mannopyranosylphenylisothiocyanate was

hydrolysed in 1 h by the addition of 25 µl Tris.HCL (pH 9.5, 1 M) per mg a-D-mannopyranosylphenylisothiocyanate used.

The mannosylated peptides were purified by gradient elution on a Jasco HPLC system, containing a Jasco PU-980 intelligent HPLC Pump, a Jasco LG-980-02 Ternary Gradient Unit, a Jasco UV-975 intelligent UV/VIS Detector, a Jasco AS-950 intelligent Sampler, controlled by a JCN Cobra Excel 486 PC using Borwin vs 1.20 for Windows (B&L Systems, Maarssen, The Netherlands) equipped with a RP-HPLC Vydac 218TP1010 column (1.0 x 25cm) using a flow of 5 ml/min and a linear gradient of water/acetonitrile/TFA 949/50/1 to water/acetonitrile/TFA 249/750/1 in 28 min. Detection was at 225 nm. The purified peptides were lyophilized in aliquots and stored at -20°C until use.

The purity of the mannosylated peptides was checked on a Waters LCM-1 system using Millenium Chromatography Manager (Waters) and equipped with a RP-HPLC Nucleosil 100-5C18AB column (0.4 x 25 cm) (Macherey-Nagel, Düren, Germany), using a flow of 1 ml/min and a linear gradient of water/acetonitrile/TFA 949/50/1 to water/acetonitrile/TFA 249/750/1 in 23 min. Detection was at 214 nm.

All mannosylated peptides were analysed by MALDI-TOF MS on a Lasermat mass spectrometer (Finnigan MAT, UK). About 5 pmol of the peptide in 0.5 µl water/acetonitrile containing 0.1% TFA was mixed on a sample slide with 0.5 µl of matrix solution (ACH, 10 mg/ml in acetonitrile/water 60/40 (v/v) containing 0.1% TFA). The sample was allowed to dry in the air for about 10 min and applied to the instrument. Calibration was performed with peptides of known molecular mass (1422.7 or 1896.1), either as external or as internal references.

The following peptides were synthesized:

K1P1	K-T-I-A-Y-D-E-E-A-R-R-G
K2P1	K-K-T-I-A-Y-D-E-E-A-R-R-G
K3P1	K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K4P1	K-K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K5P1	K-K-K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K1P1.1	K-T-I-Q-Y-D-E-E-A-R-R-G-L
K1P1.2	K-T-I-A-S-D-E-E-A-R-R-G-L
K1P2	K-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H
K1P3	K-L-Q-A-A-P-A-L-D-R-L
K1P4	K-G-G-R-G-I-L-G-F-V-F-T-L
K1P5	K-A-M-R-M-A-T-P-L-L-M-Q-A
K1P1P4	K-T-I-A-Y-D-E-E-A-R-R-G-G-R-G-I-L-G-F-V-F-T-L
M2K1P1	M-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M3K2P1	M-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M4K3P1	M-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M5K4P1	M-K(M)-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M6K5P1	M-K(M)-K(M)-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M2K1P1.1	M-K(M)-T-I-Q-Y-D-E-E-A-R-R-G-L
M2K1P1.2	M-K(M)-T-I-A-S-D-E-E-A-R-R-G-L
M2K1P2	M-K(M)-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H
M2K1P3	M-K(M)-L-Q-A-A-P-A-L-D-R-L
M2K1P4	M-K(M)-G-G-R-G-I-L-G-F-V-F-T-L
M2K1P5	M-K(M)-A-M-R-M-A-T-P-L-L-M-Q-A
M2K1P1P4	M-K(M)-T-I-A-Y-D-E-E-A-R-R-G-G-R-G-I-L-G-F-V-F-T-L

Mass spec data of the peptides:

	Formula	$MH^+_{calc(av)}$	$MH^+_{meas(av)}$
K1P1	$C_{59}H_{97}N_{19}O_{21}$	1409.5	1409.1
K2P1	$C_{65}H_{109}N_{21}O_{22}$	1537.7	1536.9
K3P1	$C_{71}H_{121}N_{23}O_{23}$	1665.9	1666.2
K4P1	$C_{77}H_{133}N_{25}O_{24}$	1794.1	1793.6
K5P1	$C_{83}H_{145}N_{27}O_{25}$	1922.2	1922.2
K1P1.1	$C_{67}H_{111}N_{21}O_{23}$	1579.7	1579.4
K1P1.2	$C_{59}H_{104}N_{20}O_{22}$	1446.6	1446.9
K1P2	$C_{90}H_{145}N_{27}O_{29}$	2070.3	2068.5
K1P3	$C_{53}H_{94}N_{16}O_{15}$	1196.4	1196.6
K1P4	$C_{65}H_{105}N_{17}O_{15}$	1365.6	n.d.
K1P5	$C_{62}H_{112}N_{18}O_{16}S_3$	1462.9	1464.0
K1P1P4	$C_{116}H_{185}N_{33}O_{33}$	2571.0	2570.0
M2K1P1	$C_{85}H_{127}N_{21}O_{33}S_2$	2036.2	2035.3
M3K2P1	$C_{104}H_{154}N_{24}O_{40}S_3$	2477.7	2476.5
M4K3P1	$C_{123}H_{181}N_{27}O_{47}S_4$	2919.2	2917.2
M5K4P1	$C_{142}H_{208}N_{30}O_{54}S_5$	3360.7	3360.9
M6K5P1	$C_{161}H_{235}N_{33}O_{61}S_6$	3802.2	3796.7
M2K1P1.1	$C_{93}H_{141}N_{23}O_{35}S_2$	2206.4	2206.2
M2K1P1.2	$C_{85}H_{134}N_{22}O_{34}S_2$	2073.2	2074.1
M2K1P2	$C_{116}H_{175}N_{29}O_{41}S_2$	2696.9	2697.3
M2K1P3	$C_{79}H_{124}N_{16}O_{27}S_2$	1823.0	1822.0
M2K1P4	$C_{91}H_{135}N_{19}O_{27}S_2$	1992.3	1992.1
M2K1P5	$C_{88}H_{142}N_{20}O_{28}S_5$	2089.5	2090.5
M2K1P1P4	$C_{142}H_{215}N_{35}O_{45}S_2$	3197.6	3195.3

Dendritic cell culture

Dendritic cells were cultured according to the protocol described by Sallusto and Lanzavecchia. Routinely the cells were cultured for 7 days. All dendritic cell cultures were checked for the expression of typical dendritic cell surface markers by fluorescence activated cell sorter analysis on day 7. The cell surface markers analysed included CD1a, HLA-class I and class II, CD4 and the mannose receptor. All cultures were found to be more than 95% positive for these markers. Subsequently the cells were frozen in a mixture of RPMI 1640 (Gibco) supplemented with 20% serum and 10% DMSO and stored in liquid nitrogen until usage.

T CELL CLONES

The T cell clones have been described in detail elsewhere (Anderson et al, Geluk et al, Oftung et al). Clone RP15.1.1 is specific for the peptide corresponding to amino acid 3-13 of Mycobacterium leprae HSP65. This peptide is designated P1. The response to the peptide is HLA-DR3 restricted. The T cell clone R3F7 is specific for the peptide corresponding to amino acid 413-424 of the Mycobacterium leprae HSP70. This peptide is designated P2. The response to the peptide is HLA-DR2 restricted. The T cell clone R2F10 is specific for the peptide corresponding to amino acid 418-427 of the Mycobacterium leprae HSP60. This peptide is designated P3. The response to the peptide is HLA-DR2 restricted. The T cell clones were expanded by specific stimulation with the appropiate peptide in the presence of autologous irradiated (3000 RAD) peripheral blood mononuclear cells as antigen presenting cells. Subsequently the cells were frozen in a mixture of RPMI 1640 supplemented with 20% serum and 10% DMSO and stored in liquid nitrogen until usage.

Proliferation assay

For the measurement of proliferation 10.000 cloned T cells were mixed with 10.000 irradiated cultured dendritic cells. To this mannosylated or non-mannosylated peptide was added in a previously determined concentration range. The total volume of the culture was 200 µl. All cultures were carried out in triplicates. Culture was at 37°C in a 5% CO_2 incubator for 48 hours. Next 1 µCi [^{3}H]-thymidine was added and the culture continued for another 16 hours. Subsequently the cultures were harvested and the [^{3}H]-thymidine incorporation was measured using a beta-plate counter (Pharmacia) using standard protocols.

RESULTS

We have investigated if the in vitro mannosylation of immunogenic peptide enhances their immunogenicity. For this purpose synthetic variants of three known antigenic peptides were synthetized with the addition of an N-terminal lysine residue. The peptides used were (single amino acid code): K-T-I-A-Y-D-E-E-A-R-R-G (designated peptide KP1), K-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H (designated peptide KP2) and K-L-Q-A-A-P-A-L-D-R-L (designated peptide KP3). The P1 peptide binds specifically to HLA-DR3 and is recognized by the human T cell clone RP15.1.1. The P2 and P3 peptide are recognized by the human T cell clones R3F7 and R2F10 respectively when bound to HLA-DR2. A portion of these peptides was bis-mannosylated by the covalent attachment of mannose to both available primary amines on the N-terminal lysine residue. The peptides are designated M-K(M)-T-I-A-Y-D-E-E-A-R-R-G (M2KP1), M-K(M)-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H (M2KP2) and M-K(M)-L-Q-A-A-P-A-L-D-R-L (M2KP3) where M stands for an covalently linked mannose moiety. Both the mannosylated and non-mannosylated forms of these peptides were purified by preparative reversed phase HPLC. The correct mannosylation was checked by mass

spectral analysis of the purified product. The exact protocol used is described herein.

Human monocyte-derived dendritic cells were cultured according to a standard protocol described above. The monocytes were isolated from the peripheral blood of an HLA-DR3 (DR17, DRbeta1*0301) positive and an HLA-DR2 positive healthy individual. The dendritic cells were co-cultured with the appropiate peptide specific T cells clones in the presence or absence of a concentration range of both the mannosylated and non-mannosylated versions of the peptides. Specific proliferation was measured by the incorporation of [^{3}H]-thymidine after 48 hours of culture. A similar experiment was performed using peripheral blood mononuclear cells as antigen presenting cells instead of dendritic cells. The exact protocol used is described herein.

In those experiments where dendritic cells were used as antigen presenting cells and the T cell clones R2F10 and RP15.1.1 as responding cells it was observed that for the induction of half maximal proliferation approximately 3,000- to 10,000-fold less of the mannosylated peptide was required compared to the non-mannosylated analog. In those experiments where dendritic cells were used as antigen presenting cells and the T cell clone R3F7 as responding cell it was observed that for the induction of half maximal proliferation approximately 100- to 300-fold less of the mannosylated peptide was required compared to the non-mannosylated analog.

In those experiments where peripheral blood mononuclear cells were used as antigen presenting cells and the T cell clones R2F10 and RP151.1 as responding cells it was observed that for the induction of half maximal proliferation approximately 1,000- to 3,000-fold less of the mannosylated peptide was required compared to the non-mannosylated analog. In those experiments where peripheral blood mononuclear cells were used as antigen presenting cells and the T cell clone R3F7 as responding cell it was observed that for the induction of half maximal proliferation approximately 3- to

10-fold less of the mannosylated peptide was required compared to the non-mannosylated analog.

It is of importance to note that the clone R3F7 requires at least 10-fold less non-mannosylated peptide for the induction of half-maximal proliferation compared to the clones R2F10 and RP15.1.1 and that the diminished effect of mannosylation in the case of the clone R3F7 may relate to the higher sensitivity of this clone.

In all experiments in which dendritic cells were used as antigen presenting cells, clone RP15.1.1 as responder cell and M2KP1 peptide, half maximal proliferation was observed at a peptide concentration of approximately 1 femtomol/ml. For the other two clones this was approximately 10 to 30 femtomol/ml. In the culture system used this corresponds to the availability of approximately 10,000 to 300,000 bis-mannosylated peptides for each dendritic cell. Since the number of mannose binding sites on cultured human dendritic cells is estimated to be 1.7×10^6 (Avrameas, A., et al. (1996)) this indicates that an absolute shortage of the mannosylated peptide resulting in suboptimal occupance of the mannose receptor on the dendritic cells directly correlates with diminished antigenic stimulation of the responding peptide specific T cells.

Next we tested the influence of multiple mannose residues on the P1 peptide on presentation by dendritic cells. For this purpose the P1 peptide was synthetized with the addition of 1, 2, 3, 4 and 5 lysine residues at the N-terminus (KP1, K2P1, K3P1, K4P1 and K5P1). These peptides were subsequently mannosylated and purified (M2KP1, M3K2P1, M4K3P1, M5K4P1 and M6K5P1) and tested for their capacity to induce proliferation of the T cell clone RP15.1.1 in the presence of DR3 positive cultured dendritic cells. Virtually identical dose response-curves were obtained with all these peptides, inducing half-maximal proliferation at a concentration of approximately 1 femtomol/ml. Thus, under these conditions the addition of more than two mannose

residues to the peptide appears not relevant for uptake and presentation by dendritic cells to T cells.

Next we tested if a short exposure of dendritic cells to a mannosylated peptide was sufficient for the induction of proliferation of the T cell clone RP15.1.1. Dendritic cells were incubated at 37°C with either P1 or M2KP1 peptide and either washed directly with excess ice cold medium (0 minute incubation time point) or after a 3 and 10 minute incubation period. Subsequently the washed dendritic cells were co-cultured with clone RP15.1.1 and the proliferation measured after 48 hours. At both the 3 and 10 minute time point both the mannosylated (M2KP1) and the non-mannosylated peptide (P1) are able to induce strong proliferation of the T cell clone. At the 0 min time point, however, only the mannosylated peptide (M2KP1) induces strong proliferation. Thus, binding of mannosylated peptide to the receptor is fast and only a short exposure of the dendritic cell to a mannosylated ligand is required for the internalisation and subsequent presentation of an immunogenic peptide.

We also examined the effect of mannosylation on the induction of recall T helper cell responses. For this purpose the proliferative response of peripheral blood mononuclear cells of an individual know to be a responder to the HSP65 3-14 peptide was determined, against the non-mannosylated and mannosylated HSP65 3-14 peptides. It was observed that for the induction of a proliferative response in these peripheral blood mononuclear cells 1000 - 10.000 fold less of the mannosylated peptide was required compared with its non-mannosylated analog. Thus, in addition to be superior in stimulating human T cell clones, the mannosylated peptides are also superior in stimulating resting T cells in peripheral blood. These results indicate that mannosylated compounds could be superior for vaccination purposes.

Finally we tested if mannosylation of peptides with antagonistic properties leads to more efficient induction of T cell non-responsiveness. Substitution analogs of peptide (K1)P1 have been described (Geluk et al., J. Immol. 149;2864,

1992) that can block the response to (K1)P1 itself. These substitution analogs therefore have antagonistic properties but could only block the response to the agonist when added in large excess (approximately 1000-fold). To test if mannosylation of these antagonists would lead to more efficient blocking we synthetized bis-mannosylated forms of these antagonists. We observed that for the blocking of the T cell response to the agonist approximately 1000-fold less of the mannosylated antagonist was needed compared the non-mannosylated antagonist. An unrelated bis-mannosylated peptide (M2K1P5) did not block the response to the agonist.

LEGEND

Table 1 gives a list of peptides which are very suitable to be used according to the present invention.
This list is by no means exhaustive or limiting.

Figure 1. Mannosylation of peptide strongly enhances its presentation by mannose receptor positive cultured human dendritic clls.

Figure 2. Mannosylation of antagonist peptide strongly enhances its presentation by mannose receptor positive cultured dendritic cells.

ABBREVIATIONS

ACH	a-cyano-4-hydroxycinnamic acid
DC	Dendritic cell
DMA	N,N-dimethylacetamide
DMSO	Dimethylsulfoxide
Fmoc	9-fluorenylmethoxycarbonyl
HSP	Heat shock protein
NMM	N-methylmorpholine
NMP	N-methylpyrrolidone
PyBop	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
TFA	Trifluoroacetic acid

Table 1

peptide	source	restriction element
VDDTQFVRFDSDAASQMEPR	HLA-A2	DR4
DLSSWTAADTAAQITQR	HLA-B62	DR4
DNPEYSPDPSIYAYD	Calreticulin	DR4

Table 1 continued

peptide	source	restriction element
NIQAQLTEQPQVTNGVQN	OMP	
TKISDFGSFIGFK	OMP	Class 1
GFKGSEDLGEGLKAV	OMP	Class 1
XGGVSVGGGASQWGN	OMP	Class 1
TLRAGRVANQFDDASQAIN	OMP	Class 1
XGGDSNNDVASQLQIFK	OMP	Class 1
XGGF\S/G\F/S\G	OMP	Class 1
XGGYYTKDTNNNL	OMP	Class 1
AVVGKPGSDVYYA	OMP	Class 1
Y/A\F/K\Y/ARHANVGRN	OMP	Class 1
XANVGRNAFELFLIGSATTSDEAKG	OMP	Class 1
DEAKGTDPLKNHQVHRLTGGY	OMP	Class 1
XGGLSENGDKAKTKNSTTE	OMP	Class 1
VPRISYAHGFDLIERGKKG	OMP	Class 1
XGGERGKKGENTSYDQ	OMP	Class 1
KRNTGIGNYTQINAA	OMP	Class 1
NYKNGGFAGNYAFKYARNHANVG		
RNAFELFLIGS		
VHRLTGGYEEGGLNLALAAQLDLS		
TKISDFGFIGFKGSELDLGD	OMP	
IGLAGEFGTRLAGRVANQFD	OMP	
YRFGNAVPRISYAHGFDFIE	OMP	
RISYAHGFDFIERGKKGENT	OMP	
YRFGNAVPRISYAHGFDFIE	OMP	
NTGIGNYTQINAASVGLRHK	OMP	

Table 1 continued

peptide		source	restriction element
AYGLDFYIL		p15	A24
QDLTMKYQIF		decapeptide	A2/A11
MLLAVLYCL		tyrosinase	A2
YMDGTMSQV		tyrosinase	A2
YMNGTMSQV		tyrosinase	
AFLPWHRLFL		tyrosinase	A24
MLLAVLYCL	1-9	tyrosinase	A2
YMNGTMSQV	369-377	tyrosinase	A2
YMDGTMSQV		tyrosinase	A2
SEIWRDIDF	192-200	tyrosinase	B44
YLEPGPVTA		gp 100/Pmel-17	A2
LLDGTATLRL		gp 100/Pmel-17	A2
KTWGQYWQV		gp 100/Pmel-17	A2
ITDQVPFSV		gp 100/Pmel-17	A2
VLYRYGSFSV		gp 100/Pmel-17	A2
KTWGQYWQV	154-162	gp 100/Pmel-17	A2
ITDQVPGSV	209-217	gp 100/Pmel-17	A2
YLEPGPVTA	280-288	gp 100/Pmel-17	A2
LLDGTATLRL	457-466	gp 100/Pmel-17	A2
VLYRYGSFSV	476-485	gp 100/Pmel-17	A2
AAGIGILTV		Melan-A/Mart-1	A2
ILTVILGVL		Melan-A/Mart-1	A2
EADPTGSHSY		Mage-1	A2
SAYGEPRKL		Mage-1	Cw*1601
EVDPIGHLY		Mage-3	A1
FLWGPRALV		Mage-3	A2
AARAVFLAL		Bage	Cw*1602
YRPRPRRY		Gage	Cw6
SPSSNRIRNT		Rage	B7
ACDPHSGHFV		CDK4 (R24 mutation)	A2
EEKLIVVLF		MUM-1 (mutated intron)	B44

Table 1 continued

peptide	source
CLLDILDTAGL	61-Leu p21ras
CLLDILDTA	wild-type p21ras
GILGFVFTL	Influenza matrix
ALWGFFGVL	Unknown (xeno-)
ALWGFFPVI	Idem
YLEPGPVTA	pMel-17 (gp100)
AAGIGILTV	Mart-1/Melan-A
AAGIGILTV	Mart-1/Melan-A
EAAGIGILTV	Mart-1/Melan-A
GILTVILGV	Mart-1/Melan-A
ALMDKSLHV	Mart-1/Melan-A
GILGFVFTL	Flu-M1
EAAGIGILTV	Melan-A
AAGIGILTV	Melan-A
YLEPGPVTA	gp100
LLDGTATLRL	gp100
KTWGQYWQV	gp100
YMNGTMSQV	Tyrosinase
MLLAVLYCL	Tyrosinase

Table 1 continued

peptide	source	restriction element
116-129	Dermatophagoides pterouyssinus p2	DR1
29-42	Dermatophagoides pterouyssinus p2	DR1 (3,4)

Table 1 continued

peptide	source	restriction elements
NP73-92	Influenza virus nucleoprot.	DR1
NP242-262	Influenza virus nucleoprot.	DR1
NP206-229	Influenza virus nucleoprot.	DR1
NP297-318	Influenza virus nucleoprot.	DR1
NP302-313	Influenza virus nucleoprot.	DR1
NP404-415	Influenza virus nucleoprot.	DR1
NP147-167	Influenza virus nucleoprot.	DR2
NP216-229	Influenza virus nucleoprot.	DR2
NP401-419	Influenza virus nucleoprot.	DR2
NP260-283	Influenza virus nucleoprot.	DR7
NP335-342	Influenza virus nucleoprot.	DQw5
NP284-391	Influenza virus nucleoprot.	B27
NP338-344	Influenza virus nucleoprot.	B37
NP338-345	Influenza virus nucleoprot.	B37
NP417-428	Influenza virus nucleoprot.	B35
SAAFEDLRVLSFIKG	Influenza A virus nucleopr.	B37
FEDLRVLS	Influenza A virus nucleopr.	B37

Table 1 continued

(Rammensee et al)

peptide	source	restriction elements
EADPTGHSY	Mage-1 161-169	A1
VSDGGPNLY	Influenza A PBI 591-599	A1
CTELKLSDY	Influenza A NP 44-52	A1
EVDPIGHLY	MAGE-3	A1
ILKEPVHGV	HIV-1 RT 476-484	A*0201
ILGFVFTLTV	Influenza matrix protein 59-68	A*0201
LLFGYPVYV	HTLV-1 tax 11-19	A*0201
GLSPTVWLSV	Hepatitis B sAg 348-357	A*0201
WLSLLVPFV	Hepatitis B sAg 335-343	A*0201
FLPSDFFPSV	Hepatitis B Nucleocapsid 18-27	A*0201
CLGGLLTMV	EBV LMP2 426-434	A*0201
FIAGNSAYEYV	HCMV glycoprotein B 618-628	A*0201
KLGEFYNQMM	Influenza B NP 85-94	A*0201
KLVALGINAV	HCV-1 1406-1415	A*0201
DLMGYIPLV	HCV core 132-140	A*0201
RLVTLKDIV	HPV 11 E7 4-12	A*0201
MLLALLYCL	Tyrosinase 1-9	A*0201
AAGIGILTV	Melan-A/Mart 1	A*0201
YLEPGPVTA	pmel 17/gp100	A*0201
ILDGTATLRL	pmel 17/gp100	A*0201
RLRDLLLIVTR	HIV-1 env gp41 768-778	A*0205
QVPLRPMTYK	HIV-1 nef 73-82	A*0205
TVYYGVPVWK	HIV-1 env gp120 36-45	A*0205
RLRPGGKKK	HIV-1 gag p17 20-29	A*0205
ILRGSVAHK	Influenza NP 265-273	A*0205
AVILPPLSPYFK	HSB 66 EST 18-29	A*1101
ASFDKAKLKK	Thymosin β-10 11-20	A*1101
IVTDFSVIK	EBNA 4 416-424	A*1101
RYLKDQQLL	HIV gp 41 583-591	A24

Table 1 continued

peptide	source	restriction element
STLPETTVVRR	Hepatitis BcAg 141-151	A*3101
IVGLNKIVR	HIV 224 gaga 267-275	A*3302
STLPETTVVRR	Hepatitis B cAg 141-151	A68.1
TPGPGVRYPL	HIV-1 nef 128-137	B7
ELRSRYWAI	Influenza NP 380-388	B8
FLRGRAYGL	EBNA 3 339-347	B8
EIYKRWIIL	HIV gagP24 262-270	B8
GEIYKRWII	HIV gagP24 261-269	B8
EIKDTKEAL	HIV gagP24 93-101	B8
SRYWAIRTR	Influenza NP 383-391	B*2705
RRRWRRLTV	EBNA LMP2 236-244	B*2705
RRYPDAVYL	Measles F protein 438-446	B*2705
RRIYDLIEL	EBNA 3C 258-266	B*2705
KRWIILGLNK	HIV-1 gag p24 265-274	B*2705
GRAFVTIGNK	HIV-1 gp120 314-322	B*2705
RRKAMFEDI	HSP 60 284-292	B*2705
KPKDELDY	P.falciparum CSP 368-375	B*3501
KSKDELDY	P.falciparum CSP 368-375	B*3501
KPNDKSLY	P.falciparum LS 1850-1857	B*3501
ASRCWVAM	HCV E1 235-242	B*3501
EDLRVLSFI	Influenza NP 339-347	B*3701
EENLLDFVRF	EBNA 6 130-139	B*4403
ILGNKIVRMY	HIV gag 267-276	B62
QMVHQAISPRTL	HIV gag 141-152	Cw*0301

Table 1 continued

SFNCGGEFF	HIV-1 gp 120 380-388	Cw*0401
PV VHFFKNIVT	MBP 98-95	DRB1*1501
PK YVKQNTLKL AT	HA 307-319	DRB1*1501
LQAAPALDKL	HSP65 418-427	DRB5*0101
VHF FKNIVTPRT P	MBP 87-99	DRB5*0101
ASD YKSAHKGFK GVD	MBP 131-145	DRB5*0101
KG FKGVDAQGT LSKI	MBP 139-153	DRB5*0101
TGHGARTSTEPTTDY	EBV gp220 592-606	DR17/DRw52
KELKRQYEKKLRQ	EBV tegument p140 1395-1407	DR17/DRw52

Table 1 continued

(Hammer et al) peptide	source	restriction element
AKFYRDPTAFGSG	Link 61-73	DR4
YNLNFHEAQQAAL	Link 170-182	DR4
DVFAFTSNFNGRF	Link 249-261	DR4
TKLTYDEAVQAAL	Link 268-280	DR4
LTLLVAAVLRAQG	Collagen 13-25	DR4
DPLQYMRADQAAG	Collagen 1165-1177	DR4
QYMRADQAAGGLR	Collagen 1168-1180	DR4
MTFLRLLSTEGSQ	Collagen 1306-1318	DR4
TFLRLLSTEGSQN	Collagen 1307-1319	DR4
KALLIQGSNDVEI	Collagen 1340-1352	DR4
VEIRAEGNSRFTY	Collagen 1350-1362	DR4
FGLQLELTEGMRF	hsp65 181-193	DR4
EGMRFDKGYISGY	hsp65 189-201	DR4
PYILLVSSKVSTV	hsp65 216-228	DR4
KAMLQDMAILTGG	hsp65 284-296	DR4
GLFLTTEAVVADK	hsp65 509-521	DR4
YILFAHLVNKVHI	Fillagrin 9-21	DR4
FAIINLFNEYSKK	Fillagrin 34-46	DR4
GSFLYQVSTHEQS	Fillagrin 491-503	DR4
SFLYQVSTHEQSE	Fillagrin 492-504	DR4
SSFSQDRDSQAQS	Fillagrin 644-656	DR4
GSFLYQVSTHEQS	Fillagrin 815-827	DR4
SFLYQVSTHEQSE	Fillagrin 816-828	DR4
GSFIYQVSTHEQS	Fillagrin 1139-1151	DR4
SFIYQVSTHEQSS	Fillagrin 1140-1152	DR4

Table 1 continued

(Vogt et al) peptide	source	restriction element
PKYVKQNTLKLAT	HA (307-319)	DRB5*0101
LQAAPALDKL	HSP65 (418-427)	DRB5*0101
PVVHFFKNIVTPRTPPPSQGK	MBP (85-105)	DRB5*0101
ASDYKSAHKGFKGVD	MBP (131-154)	DRB5*0101
KGFKGVDAQGTLSKI	MBP (139-153)	DRB5*0101
PVVHFFKNIVTPRTPPPSQGK	MBP (82-105)	DRB5*0101
GTLSKIFKLGGRDSRSG	MBP (148-162)	DRB5*0101

Table 1 continued

(Chicz et al) peptide	source	restriction elements
ATSTKKLHKEPATLIKAIDG	NASE 1-20	DR1
PATLIKAIDGDTVKLMYKGQ	NASE 11-30	DR1
DRVKLMYLGQPMTGFLLVD	NASE 21-40	DR1
VAYVYKPNNTHEQHLRRKSEA	NASE 111-130	DR1
QKQEPIDKELYPLTSL	HIV p13 97-112	DR1
GARASVLSGGLDKWE	HIV p17 1-16	DR1
RTLYQNVGTYVSVGTSTLNK	Influenza HA 187-206	DR1
PKYVKQNTLKLAT	Influenza HA 307-319	DR1
LKKLVFGYRKPLNDI	P.falciparum p190 249-263	DR1
KHIEQYLKKIKNS	P.falciparum CS 329-341	DR1
GRTQDENPVVHFFKNIVTPRTPPP	Myelin basic protein 75-98	DR1
PLKAEIAQRLEDV	Influenza matrix 19-31	DR1
RQILGQLQPSLQTGSE	HIV p17 57-72	DR1
INTKCYKLEHPVTGCG	PLA_2 85-100	DR1
YKLNFYFDLLRAKL	P.falciparum P190 211-224	DR1
ERFAVNPGLLERSEGC	HIV p17 41-56	DR1
EALVRQLAKVAYVYKPNNT	NASE 101-120	DR1
PIVQNLQGQMVHQAIS	HIV p25 1-16	DR1
KMYFNLINTKCYKLEH	PLA_2 79-94	DR1

Table 1 continued

(Geluk et al) peptide	source
LPKPPKPVSKMRMATPLLMQALPM	Ii p80-103
PKYVKQNTLKLAT	HA p308-319
KTIAYDEEARR	hsp65 p3-13

Table 1 continued

peptide	source	restriction elements
(Wang et al)		
MSLQRQFLR	gp75	
(Gouilloux et al)		
VLPDVFIRC	N-acetylglucosaminyltransferase	A2

Table 1 continued

peptide	source	restriction elements
KLPDLCTEL	HPV 18 E6 (13-21)	A2.1
SLQDIEITC	HPV 18 E6 (24-32)	A2.1
LQDIEITCV	HPV 18 E6 (25-33)	A2.1
EITCVYCKT	HPV 18 E6 (29-37)	A2.1
KTVLELTEV	HPV 18 E6 (36-44)	A2.1
ELTEVFEFA	HPV 18 E6 (40-48)	A2.1
FAFKDLFVV	HPV 18 E6 (47-55)	A2.1
DTLEKLTNT	HPV 18 E6 (88-96)	A2.1
LTNTGLYNL	HPV 18 E6 (93-101)	A2.1
TLQDIVLHL	HPV 18 E7 (7-15)	A2.1
FQQLFLNTL	HPV 18 E7 (86-94)	A2.1
QLFLNTLSF	HPV 18 E7 (88-96)	A2.1
LFLNTLSFV	HPV 18 E7 (89-97)	A2.1
LSFVCPWCA	HPV 18 E7 (97-102)	A2.1
YRDGNPYAV	HPV 16 E6 (61-69)	A1
WTGRCMSCC	HPV 16 E6 (139-147)	A1
MSCCRSSRT	HPV 16 E6 (144-152)	A1
TTDLYCYEQ	HPV 16 E7(19-27)	A1
EIDGPAGQA	HPV 16 E7 (37-45)	A1
HVDIRTLED	HPV 16 E7 (73-81)	A1
AMFQDPQER	HPV 16 E6 (7-15)	A2.1
KLPQLCTEL	HPV 16 E6 (18-26)	A2.1
QLCTELQTT	HPV 16 E6 (21-29)	A2.1
LCTELQTTI	HPV 16 E6 (22-30)	A2.1
ELQTTIHDI	HPV 16 E6 (25-33)	A2.1
LQTTIHDII	HPV 16 E6 (26-34)	A2.1
TIHDIILEC	HPV 16 E6 (29-37)	A2.1
IHDIIECV	HPV 16 E6 (30-38)	A2.1
CVYCKQQLL	HPV 16 E6 (37-45)	A2.1
FAFRDLCIV	HPV 16 E6 (52-60)	A2.1
KISEYRHYC	HPV 16 E6 (79-87)	A2.1
PLCDLLIRC	HPV 16 E6 (102-110)	A2.1
TLHEYMLDL	HPV 16 E7(7-15)	A2.1

Table 1 continued

YMLDLQPET	HPV 16 E7 (11-19)	A2.1
MLDLQPETT	HPV 16 E7 (12-20)	A2.1
RLCVQSTHV	HPV 16 E7 (66-74)	A2.1
TLEDLLMGT	HPV 16 E7 (78-86)	A2.1
LLMGTLGIV	HPV 16 E7 (82-90)	A2.1
GTLGIVCPI	HPV 16 E7 (85-93)	A2.1
TLGIVCPIC	HPV 16 E7 (86-94)	A2.1
AMFQDPQER	HPV 16 E6 (7-15)	A3.2
IILECVYCK	HPV 16 E6 (33-41)	A3.2
CVYCKQQLL	HPV 16 E6 (37-45)	A3.2
VYCKQQLLR	HPV 16 E6 (38-46)	A3.2
QQLLRREVY	HPV 16 E6 (42-50)	A3.2
IVYRDGNPY	HPV 16 E6 (59-67)	A3.2
YAVCDKCLK	HPV 16 E6 (67-75)	A3.2
AVCDKCLKF	HPV 16 E6 (68-76)	A3.2
VCDKCLKFY	HPV 16 E6 (69-77)	A3.2
KFYSKISEY	HPV 16 E6 (77-83)	A3.2
KISEYRHYC	HPV 16 E6 (79-87)	A3.2
ISEYRHYCY	HPV 16 E6 (80-88)	A3.2
RHYCYSLYG	HPV 16 E6 (84-92)	A3.2
SLYGTTLEQ	HPV 16 E6 (89-97)	A3.2
TTLEQQYNK	HPV 16 E6 (93-101)	A3.2
QQYNKPLCD	HPV 16 E6 (97-105)	A3.2
LIRCINCQK	HPV 16 E6 (107-115)	A3.2
HLDKKQRFH	HPV 16 E6 (125-133)	A3.2
CMSCCRSSR	HPV 16 E6 (143-151)	A3.2
SCCRSSRTR	HPV 16 E6 (145-153)	A3.2
CCRSSRTRR	HPV 16 E6 (146-154)	A3.2
HYNIVTFCC	HPV 16 E7 (51-59)	A3.2
YNIVTFCCK	HPV 16 E7 (52-60)	A3.2
CCKCDSTLR	HPV 16 E7 (58-66)	A3.2
KCDSTLRLC	HPV 16 E7 (60-68)	A3.2
AMFQDPQER	HPV 16 E6 (7-15)	A11.2
IILECVYCK	HPV 16 E6 (33-41)	A11.2
CVYCKQQLL	HPV 16 E6 (37-45)	A11.2

Table 1 continued

VYCKQQLLR	HPV 16 E6 (38-46)	A11.2
QQLLRREVY	HPV 16 E6 (4-50)	A11.2
IVYRDGNPY	HPV 16 E6 (59-67)	A11.2
YAVCDKCLK	HPV 16 E6 (67-75)	A11.2
AVCDKCLKF	HPV 16 E6 (68-76)	A11.2
VCDKCLFKY	HPV 16 E6 (69-77)	A11.2
KISEYRHYC	HPV 16 E6 (79-87)	A11.2
ISEYRHYCY	HPV 16 E6 (80-88)	A11.2
LIRCINCQK	HPV 16 E6 (107-115)	A11.2
TGRCMSCCR	HPV 16 E6 (140-148)	A11.2
CMSCCRSSR	HPV 16 E6 (143-151)	A11.2
SCCRSSRTR	HPV 16 E6 (145-153)	A11.2
HYNIVTFCC	HPV 16 E7 (51-59)	A11.2
YNIVTFCCK	HPV 16 E7 (52-60)	A11.2
CCKCDSTLR	HPV 16 E7 (58-66)	A11.2
VCPICSQKP	HPV 16 E7 (90-98)	A11.2
MHQKRTAMF	HPV 16 E6 (1-9)	A24
LQTTIHDII	HPV 16 E6 (26-34)	A24
VYCKQQLLR	HPV 16 E6 (38-46)	A24
LLRREVYDF	HPV 16 E6 (44-52)	A24
VYDFAFRDL	HPV 16 E6 (49-57)	A24
PYAVCDKCL	HPV 16 E6 (66-74)	A24
KCLKFYSKI	HPV 16 E6 (72-80)	A24
EYRHYCYSL	HPV 16 E6 (82-90)	A24
HYCYSLYGT	HPV 16 E6 (85-93)	A24
CYSLYGTTL	HPV 16 E6 (87-95)	A24
RFHNIRGRW	HPV 16 E6 (131-139)	A24
RAHYNIVTF	HPV 16 E7 (49-57)	A24

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CLAIMS

1. A method for enhancing uptake of a proteinaceous antigen or a peptide derived therefrom by antigen presenting cells having a mannose or similar receptor, whereby the antigen or peptide is provided with at least one and preferably two mannose groups or a functional equivalent thereof.

2. A method for enhancing antigen presentation by an antigen presenting cell comprising contacting said cell with a soluble antigen which has been provided with at least one and preferably two mannose groups or a functional equivalent thereof and allowing the cell to internalize and process said mannosylated antigen.

3. A method for producing a cell presenting a peptide antigen, whereby said peptide antigen is provided with at least one and preferably two mannose groups or a functional equivalent thereof, comprising contacting said cell with a soluble antigen which has been provided with a mannose group or a functional equivalent thereof and allowing the cell to internalize and process said mannosylated antigen.

4. A method according to claims 1-3 whereby the antigen presenting cell is a cell which presents peptides in the context of MHC-class II and/or MHC-class I.

5. A method according to claims 1-4 whereby the antigen presenting cell is a dendritic cell.

6. An antigen presenting cell obtainable by a method according to claim 3.

7. A cell according to claim 6 which is a dendritic cell.

8. A method for improving the T cell response to a peptide antigen comprising contacting said T cell with a cell according to claim 6 or 7.

9. A vaccine preparation for the treatment or prophylaxis of infection by a pathogen comprising at least one proteinaceous antigen derived from said pathogen, which proteinaceous antigen is provided with at least one and

preferably two mannose groups or a functional equivalent thereof.

10. A vaccine preparation for the treatment or prophylaxis of infection by a pathogen comprising at least one peptide derived from said pathogen which peptide is provided with at least one and preferably two mannose groups or a functional equivalent thereof.

11. A peptide provided with at least one and preferably two mannose groups or a functional equivalent thereof for use in the preparation of a vaccine.

12. A proteinaceous antigen provided with at least one and preferably two mannose groups or a functional equivalent thereof for use in the preparation of a vaccine.

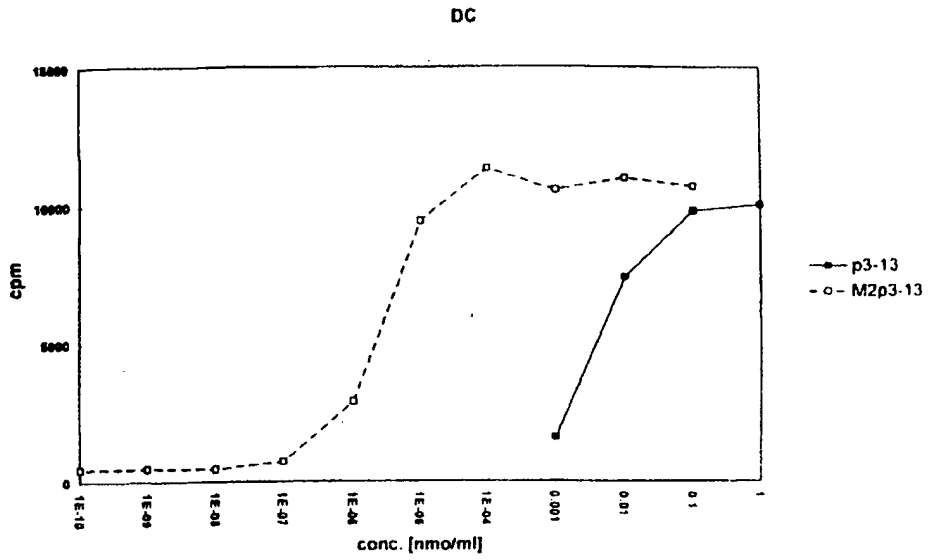
DC
cpm
conc. [nmo/ml]
p3-13
M2p3-13

Fig. 1

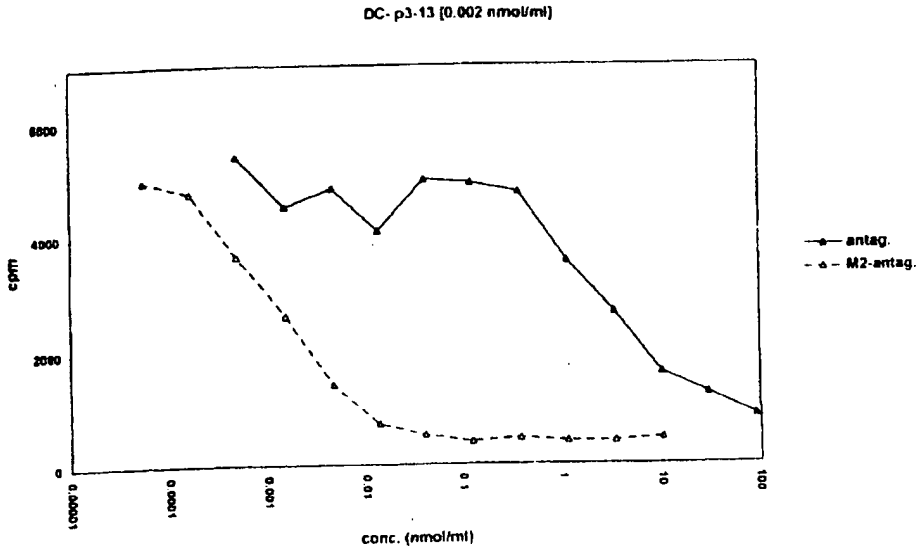
DC- p3-13 [0.002 nmol/ml]
cpm
conc. (nmol/ml)
antag.
M2-antag.

Fig. 2

INTERNATIONAL SEARCH REPORT

Inte .ional Application No
PCT/NL 97/00536

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/35 A61K39/04

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. LANZAVECCHIA: "Mechanisms of uptake for presentation" CURRENT OPINION IN IMMUNOLOGY, vol. 8, no. 3, June 1996, LONDON GB, pages 348-354, XP002028910 see page 349, left-hand column, paragraph 3 see page 349, left-hand column, paragraph 5 - right-hand column, paragraph 1 --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
23 January 1998	12/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/NL 97/00536

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	F. SALLUSTO ET AL.: "Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 2, August 1995, pages 389-400, XP000647847 see page 396, right-hand column, paragraph 3 - page 399, right-hand column, paragraph 2 ---	1-11
A	R.M. STEINMAN AND J. SWANSON: "The Endocytic Activity of Dendritic cells" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 2, 1 August 1995, pages 283-288, XP000647848 see page 284, right-hand column, last paragraph - page 285, left-hand column, paragraph 1 ---	1-11
A	EP 0 659 768 A (AUSTIN RESEARCH INST) 28 June 1995 see claims; examples ---	1-11
P,X	F. KONING: "Enhancement of HLA class II restricted antigen presentation by mannose-receptor-mediated uptake" BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 25, no. 2, May 1997, pages 664-665, XP002053241 see the whole document -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte .ional Application No
PCT/NL 97/00536

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EP 0659768 A	28-06-95	AU 1308195 A	17-07-95
		AU 8172894 A	29-06-95
		WO 9518145 A	06-07-95
		CA 2135833 A	25-06-95
		JP 7206707 A	08-08-95